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The *Neurospora* circadian clock: simple or complex?

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The fungus *Neurospora crassa* is being used by a number of research groups as a model organism to investigate circadian (daily) rhythmicity. In this review we concentrate on recent work relating to the complexity of the circadian system in this organism. We discuss: the advantages of *Neurospora* as a model system for clock studies; the frequency (*frq*), *white collar-1* and *white collar-2* genes and their roles in rhythmicity; the phenomenon of rhythmicity in null *frq* mutants and its implications for clock mechanisms; the study of output pathways using clock-controlled genes; other rhythms in fungi; mathematical modelling of the *Neurospora* circadian system; and the application of new technologies to the study of *Neurospora* rhythmicity. We conclude that there may be many gene products involved in the clock mechanism, there may be multiple interacting oscillators comprising the clock mechanism, there may be feedback from output pathways onto the oscillator(s) and from the oscillator(s) onto input pathways, and there may be several independent clocks coexisting in one organism. Thus even a relatively simple lower eukaryote can be used to address questions about a complex, networked circadian system.

Keywords: *Neurospora*; circadian; clock; oscillator; frequency gene; white-collar genes

1. INTRODUCTION

Circadian (daily) clocks provide organisms with a time-keeping ability that confers an adaptive advantage and has no doubt contributed to the ubiquity of circadian clocks in the biological world. A 'circadian rhythm' is one that has the following properties (for a review, see Edmunds 1988): a self-sustained endogenous oscillation under constant conditions; a period close to 24 h; the ability to be entrained by environmental signals, primarily light and temperature cycles; and temperature compensation of the period. These circadian properties might be intrinsic to a single central oscillator mechanism, or they might emerge from interactions between an oscillator and input pathways or other oscillator(s). Throughout this review the term 'oscillator' refers to a self-sustained rhythm generator, while a 'rhythm' is any observable oscillation in a variable. The 'circadian oscillator' is the mechanism that, under normal circumstances, generates a rhythmic output that satisfies the criteria listed above. (Under certain circumstances such as in mutant strains or unusual environmental conditions, this mechanism might produce rhythmic output that is not circadian in period, nor sustainability, nor temperature

compensation, nor light sensitivity.) We will consider 'the clock' to be the entire intact, integrated circadian system as it is used by the organism for tracking the passage of time, including the input and output pathways.

In higher organisms, the intact circadian system is considered to be the product of crosstalk between many integrated oscillating pathways (Roenneberg & Merrow 2001). However, despite this level of complexity, the basis of rhythmicity in all organisms lies within the cell (e.g. Welsh *et al.* 1995). Consequently, two distinct aspects of circadian rhythms need to be addressed: (i) what are the functional components of the cell required for rhythmicity? and (ii) how do the cellular components communicate time information between cells or within a multicellular organism? Using a model organism such as *Neurospora crassa* that can be manipulated with ease allows us to dissect an inherently complex, networked system and to formulate questions regarding the influences of the environment and metabolic state of the cell on oscillatory behaviour.

This review culminated from an interesting and provocative meeting on Complex Clocks held at the University of Edinburgh in March 2000 on the occasion of Professor David Saunders' retirement. Here, we attempt to summarize what is known about the time-keeping mechanism of *Neurospora* and to incorporate information regarding the complexity of its circadian system.

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All authors contributed in equal part to this work.

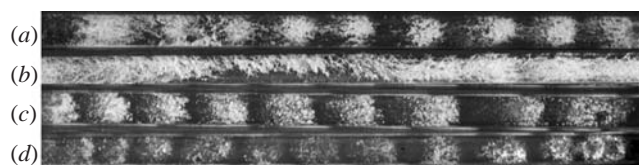


Figure 1. The conidial banding rhythm of *Neurospora crassa*. Colonies are grown on solid agar medium in glass 'race tubes' about 30 cm long. Tubes are inoculated at one end with conidiospores (asexual spores) and after an initial exposure to light the cultures are grown in constant darkness and constant temperature. Cultures are observed once a day under a red safe-light to mark the extent of growth. Growth is from left to right. All strains carry the *bd* mutation which makes conidiation resistant to high levels of CO₂ and allows the expression of the conidial banding rhythm in closed culture vessels, and the *csp-1* mutation which prevents the release of conidiospores but has no major effect on rhythmicity. The *frq*¹⁰ mutation is a null mutation at the *frq* locus and this strain does not produce FRQ protein. The *chol-1* strain is defective in lipid synthesis and requires choline for normal growth and rhythmicity. Under the conditions shown here, without choline supplementation, growth of *chol-1* strains is slow and the period of the conidial banding rhythm is about 60 h. (a) *frq*⁺, (b) *frq*¹⁰, (c) *frq*⁺ *chol-1*, (d) *frq*¹⁰ *chol-1*.

2. NEUROSPORA CRASSA AS A MODEL SYSTEM FOR STUDIES OF THE CIRCADIAN CLOCK

One of the most appealing properties of *Neurospora* as a model clock organism is that it is remarkably convenient to handle in the laboratory. The standard assay for the conidiation rhythm (figure 1a) does not require expensive monitoring equipment or labour-intensive procedures (reviewed by Bell-Pedersen *et al.* 1996b). As hyphae grow across an agar surface, once a day the clock signals the production of the easily observable fluffy orange conidiospores. Under constant conditions in the absence of environmental time-cues, these bands of spores occur with a circadian period, approximately every 21–22 h. The pattern of conidiation bands produced can be analysed later at leisure, and acts as a 'fossil record' of the state of the clock at the time the growth front was produced. The conidial banding rhythm in the wild-type fulfils all the requirements of a circadian rhythm, as reviewed by Bell-Pedersen *et al.* (1996b).

Neurospora has been a model organism in genetics laboratories since the 1920s (Perkins 1992) and the wealth of mutants and techniques is another of its major advantages for rhythm studies. It is a haploid organism, so the phenotypes of mutations are immediately visible and new mutations are easily selected. Heterokaryons can be used to determine dominance and to maintain recessive lethal mutations. It has a short generation time, sexual crosses are simple, and many progeny are produced from one cross. Thousands of single- and multiple-mutant strains have been established over the years and are maintained readily available to the *Neurospora* community by the Fungal Genetics Stock Center (<http://www.fgsc.net>). There is also a largely untapped resource of thousands of wild-collected strains, maintained by Perkins (Perkins & Turner 1988). A survey of clock phenotypes in this collection might reveal new clock-affecting loci and could be used to answer questions about the adaptive value and evolutionary origins of clocks. Preliminary work on rhythmicity in wild-collected strains of *Neurospora intermedia* has been reported by Morgan & Feldman (1998).

Although *Neurospora* was slow to enter the modern era of molecular biology, molecular tools are now available (Hynes 1996). The organism does not support autonomously replicating plasmids, but transformation by chromosomal insertion is routine. Genes can be targeted

to specific loci, and inducible promoters which are unregulated by the circadian system are available for regulating transgenes. Genes can be inactivated by repeat-induced point mutations (RIP) (Irelan & Selker 1996) or by gene replacement (Aronson *et al.* 1994c). There is an ongoing *Neurospora* genome and proteome project involving a number of laboratories in two countries: (<http://biology.unm.edu/biology/ngp/home.html>, <http://www.genome.ou.edu/fungal.html> and <http://www.mips.biochem.mpg.de/proj/neurospora/>), and the complete *Neurospora* genome sequence is now available (<http://www-genome.wi.mit.edu/>).

The field of biochemical genetics developed from the isolation of the first nutritional mutants in *Neurospora* by Beadle & Tatum in 1941 (cited by Perkins 1992). Subsequent work with *Neurospora* helped define many biochemical pathways and has given us a wealth of metabolic mutants. *Neurospora* biochemistry is similar to that of higher eukaryotes such as animals but without the complications of many differentiated tissue types and multicellular interactions. These advantages make *Neurospora* valuable for biochemical analysis of the functions of clock gene products and the kinetics of the reactions in which they participate.

Model systems such as *Neurospora* have the potential to allow us to tackle problems that would be intractable in more complex organisms, with the hope that we will be able to apply what we learn to organisms of more intrinsic interest, such as humans. Many of the formal properties of circadian clocks are conserved across species and some aspects of the molecular mechanism are also conserved (reviewed in Dunlap 1999). However, we should also remember that although we value *Neurospora* as a model organism, much of its behaviour might be shaped by the very specific ecological demands on an organism with indeterminate growth coping with a heterogeneous environment. In other words, we should not forget that *Neurospora* is not just a very flat fruit fly or a particularly stupid mammal but a unique fungus with a proud history and an exciting future.

3. WHAT IS KNOWN ABOUT CIRCADIAN OSCILLATOR COMPONENTS IN NEUROSPORA?

Genetic screens have identified a number of molecules and processes that affect the *Neurospora* circadian clock

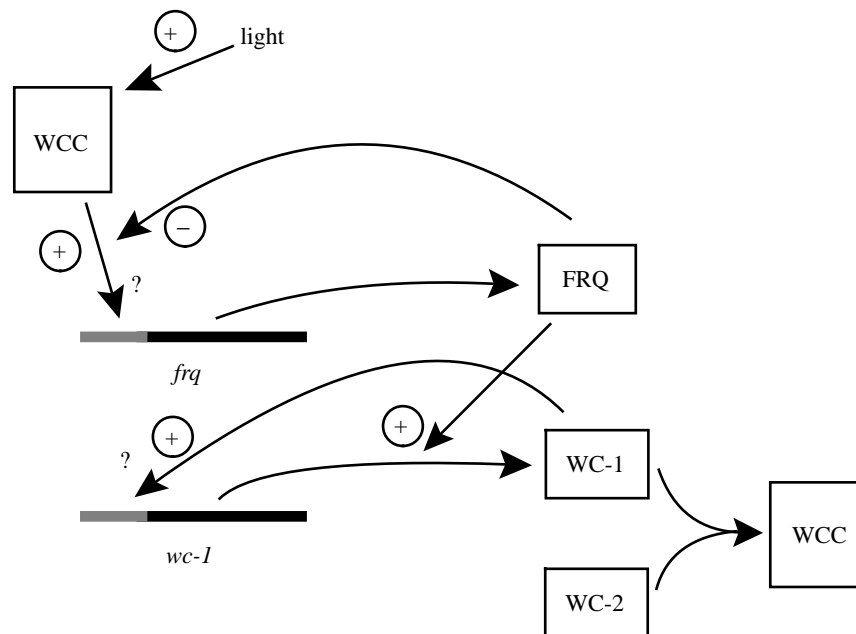


Figure 2. Regulation of expression of the *frequency* and *white collar* genes in *Neurospora*. *frq*, *frequency* gene; *wc-1*, *white collar-1* gene; FRQ, protein product of the *frequency* gene; WC-1 and WC-2, protein products of the *white collar* genes; WCC, white collar complex. Regulatory interactions are described in the text. Question marks indicate interactions that are still somewhat speculative—it has been proposed that FRQ regulates its own transcription through interaction with the WCC (Crosthwaite *et al.* 1997), but transcriptional regulation of *frq* expression has not been directly demonstrated. Similarly, regulation of *wc-1* expression by WC-1 has been proposed as a possibility (Morrow *et al.* 2001) but not directly demonstrated.

(Feldman & Hoyle 1973; Lakin-Thomas *et al.* 1990; Dunlap 1993). The *frequency* (*frq*) and *white collar* (*wc*) loci have been shown to encode integral components of a molecular feedback loop that is essential for the circadian properties of rhythmicity in *Neurospora*. Their products regulate activation and repression of their own expression through transcriptional and post-transcriptional control mechanisms (summarized in figure 2).

frq was isolated in a genetic screen for period mutants (Feldman & Hoyle 1973) and was the first *Neurospora* clock gene to be cloned (McClung *et al.* 1989). The *frq* locus encodes several overlapping sense and antisense transcripts. The sense transcripts contain a large open reading frame (ORF) which encodes two forms of FRQ protein (Aronson *et al.* 1994a; Garceau *et al.* 1997; Liu *et al.* 1997). Mutations in FRQ alter the period and temperature compensation properties of the clock (Gardner & Feldman 1981) and expression levels and localization of FRQ correlate with specific times of day (Aronson *et al.* 1994b; Luo *et al.* 1998). Two antisense transcripts are also produced; however, no long ORFs have been identified in these transcripts and experiments to define their significance are underway (S. Crosthwaite, unpublished data).

Molecular hallmarks of circadian rhythmicity in *Neurospora* include *frq* mRNA and FRQ protein, which cycle in abundance with circadian periodicity. The transcript peaks mid-morning (Aronson *et al.* 1994b) with the FRQ protein peaking 6–8 h later (Garceau *et al.* 1997). Early work produced evidence that one function of FRQ protein was to repress accumulation of its own transcript, since in an arrhythmic, null mutant strain (*frq*⁰) the level of *frq* RNA is high and arrhythmic (Aronson *et al.* 1994b).

Furthermore, artificial induction of FRQ results in a decrease of *frq* mRNA. Mutations in either of the putative transcription factors, WC-1 and WC-2, result in low levels of *frq* RNA and FRQ protein (Crosthwaite *et al.* 1997), suggesting that the cyclic accumulation of *frq* mRNA is transcriptionally controlled either directly or indirectly by WC-1 and WC-2. FRQ may act to repress its expression by direct interaction with these proteins and in support of this, interaction between FRQ and WC-2 has been demonstrated *in vitro* and *in vivo* (Denault *et al.* 2001; Morrow *et al.* 2001).

Consistent with the suggested role of FRQ in transcriptional repression, nuclear localization of FRQ is essential for overt rhythmicity and, coincident with transport of FRQ to the nucleus, *frq* mRNA levels decrease (Luo *et al.* 1998). FRQ enters the nucleus as soon as it is made and represses accumulation of *frq* mRNA within 3–6 h. Time to derepression of *frq* expression takes the remainder of the cycle (16 h) and correlates with turnover of FRQ protein (Morrow *et al.* 1997). FRQ, which is progressively phosphorylated through the day, disappears shortly after hyperphosphorylated forms are detected. Moreover, inhibition of phosphorylation results in a decreased rate of FRQ turnover and an increase in period length (Liu *et al.* 2000). The kinase responsible has not yet been identified but obvious parallels between clocks in different organisms suggest casein kinase I as a candidate (Kloss *et al.* 1998). The pattern of FRQ accumulation and its phosphorylation state in the nucleus is approximately 3 h advanced compared with that of FRQ in the cytoplasm (Luo *et al.* 1998). This difference in profile suggests nuclear entry as a major time reference point in the cycle.

frq has been an indispensable tool, not only for investigating the clock mechanism but also for elucidating the molecular effects of entraining stimuli on the clock and the identification of mutants with defects in a light input pathway to the clock (Aronson *et al.* 1994b; Crosthwaite *et al.* 1995, 1997; Liu *et al.* 1997; Merrow *et al.* 1999). For instance, the WC proteins, though first identified as global regulators of light-regulated processes (Degli-Innocenti & Russo 1984), were subsequently shown to be necessary for a functional circadian clock. In WC-1 or WC-2 mutant strains, the conidiation rhythm is abolished and expression of *frq* in the dark is low and arrhythmic (Crosthwaite *et al.* 1997). Furthermore, as discussed in the next section, in the absence of FRQ there is a complete loss of synchronization of conidiation by light (Chang & Nakashima 1997; Merrow *et al.* 1999; Lakin-Thomas & Brody 2000). Thus, the *Neurospora* oscillator mechanism is tightly linked to the light input pathway (Crosthwaite *et al.* 1997; Merrow *et al.* 2001).

In contrast to *frq* mRNA, *wc-1* and *wc-2* transcripts are apparently not rhythmically expressed (Dunlap 1999; Lee *et al.* 2000; Merrow *et al.* 2001). Nevertheless, WC-1 protein is produced rhythmically 180° out of phase relative to FRQ, reaching peak levels in the subjective night (Lee *et al.* 2000; Merrow *et al.* 2001). Recent data indicate that this expression pattern is a consequence of FRQ acting at a post-transcriptional level to promote accumulation of WC-1 protein at certain times of day (Lee *et al.* 2000). Thus, FRQ triggers both activation and repression of gene expression, which may strengthen the amplitude of the rhythm.

The WC proteins exist *in vitro* as homodimers and both *in vitro* and *in vivo* as heterodimers in a white collar complex (WCC) through interaction between their PAS domains (Ballario *et al.* 1998; Talora *et al.* 1999). PAS-domain-containing proteins, conserved in eubacteria, archaeobacteria and eukaryotes, encompass known regulators of development and physiological processes, including proteins that are involved in the detection of and adaptation to environmental signals such as light (Taylor & Zhulin 1999). WC-1 is present almost exclusively in the nucleus whereas WC-2 is both nuclear and cytoplasmic. Nuclear localization is independent of both WC-1/WC-2 heterodimerization and light (Schwerdtfeger & Linden 2000). What then is the trigger that enables WC activation of gene expression? Both proteins are phosphorylated in response to light, and one suggestion is that phosphorylation alters their activity (Talora *et al.* 1999; Schwerdtfeger & Linden 2000). Indeed, the kinetics of WC-1 phosphorylation correlate with light-induced gene expression (Schwerdtfeger & Linden 2000). However, there is no evidence to date of rhythmic phosphorylation of WC-1 (Lee *et al.* 2000). Therefore, from the available evidence, it seems unlikely that the same mechanism considered to confer general light responsiveness is used by endogenous signals to drive rhythmic *frq* expression in constant darkness (DD).

Investigations of the effect of light and temperature on *frq* expression have aided our understanding of entrainment phenomena. Light pulses given to dark grown *Neurospora* result in a rapid, dose-dependent accumulation of *frq* RNA at all times of day, i.e. light induction overrides FRQ-induced negative feedback (Crosthwaite *et al.*

1995). A pulse of light given early in the subjective day, when *frq* mRNA levels are rising, advances the phase of the rhythm, whereas a pulse of light given late in the day, when *frq* mRNA levels are falling, results in a phase delay. Saturating amounts of light given at any time during the subjective day have the same effect on overall levels of *frq* mRNA and FRQ protein and reset the circadian phase to between mid-morning and dusk.

In contrast to light, temperature does not affect *frq* transcript levels but works at a post-transcriptional level to alter both the form and level of FRQ protein produced (Liu *et al.* 1998). The sense transcripts encode two forms of the FRQ protein, a long form (LFRQ) and a shorter form (SFRQ) (Garceau *et al.* 1997). At high temperatures, there is proportionally more LFRQ (Liu *et al.* 1997). This temperature-dependent regulation of translation is required for the conidiation rhythm over a range of physiological temperatures. However, either form of FRQ alone, if made in sufficient quantities, is able to sustain rhythmicity within a certain temperature range (Liu *et al.* 1998). A change in temperature initially resets the phase of the oscillator. However the system quickly adapts to the new temperature with FRQ oscillating around a higher or lower absolute level with no change in periodicity.

4. WHAT DOES THE PHENOTYPE OF THE FRQ NULL MUTANTS MEAN AND HOW CAN IT BE INTERPRETED?

Robust, self-sustained rhythmicity is lost in the absence of the FRQ protein, at least under conditions typically used for screening rhythms in *Neurospora* (Loros & Feldman 1986; Aronson *et al.* 1994a). Rather than asexual spore formation every 22 h in constant darkness, FRQ-deficient strains fill the race tube with a relatively even (though generally sparse) lawn of conidia (figure 1b). The *frq⁹* mutation results in a truncated protein (Aronson *et al.* 1994a) that has only recently been immunochemically identified, although no function for this protein has yet been described (M. Merrow, M. Görl, Z. Dragovic and T. Roenneberg, unpublished data). Subsequently a *frq* gene replacement was produced by homologous recombination (Aronson *et al.* 1994a). The clock phenotype of these two *frq* mutants (*frq⁹* and *frq¹⁰*, respectively) is indistinguishable.

However, under certain circumstances FRQ-deficient strains are rhythmic as judged by conidiation. When they are placed on extra-long race tubes a conidiation rhythm sometimes commences after approximately 5 days. Though the period can be around 24 h in length (circadian), it can also show a range of periods from 12 h to 30 h, depending on the medium and temperature. Thus the rhythmicity is neither temperature- nor nutritionally-compensated (Aronson *et al.* 1994a; Loros & Feldman 1986). A second example is seen when the null *frq* mutation is combined with various mutations in lipid metabolism (e.g. *cel* or *chol-1*, figure 1c,d) (Lakin-Thomas & Brody 2000). When the single-mutant *cel* or *chol-1* strains are lipid-deficient, periodicity is well outside the circadian range (from 35 h to 100 h, depending on conditions). However, when the medium is titrated with appropriate nutritional supplements, the period titrates to around 22 h. In double mutant strains carrying either *cel* or *chol* mutations and

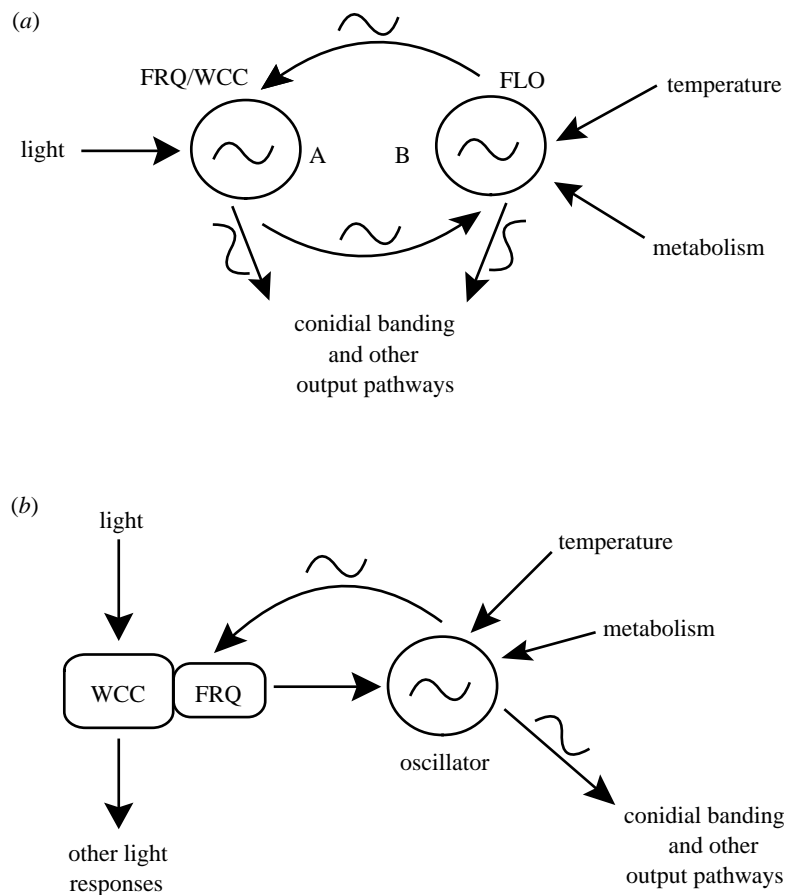


Figure 3. Alternative models for the *Neurospora* circadian system. Arrows indicate regulatory interactions, which may be transcriptional or post-transcriptional. Squiggles indicate rhythmic signals. WCC, white collar complex; FRQ, protein product of the *frequency* gene; FLO, *frq*-less oscillator. For detailed descriptions see text. (a) A two-oscillator system with the A and B oscillators mutually coupled. (b) A one-oscillator system in which FRQ and WCC are located in the light input pathway.

frq-null mutations, the period also titrates to 22 h before rhythmicity is lost with full supplementation. The systematic restoration of circadian period in parallel with complementation of the metabolic defect (by supplementation of the media) suggests that the long period rhythmicity in *cel* and *chol-1* may be related to the circadian rhythm in the intact strain.

Where does this 'residual' rhythmicity derive from? Why or how can it be conditional? In the long race tubes perhaps *Neurospora* effectively conditions its environment, creating a microenvironment compatible with fostering oscillations which are otherwise damped or of low amplitude and not normally visualized. In support of this, preliminary evidence indicates that cultures of *Neurospora* secrete substances that condition the media (M. Merrow and T. Roenneberg, unpublished data).

There are circadian properties distinct from those required for self-sustained rhythmicity, specifically entrainment, and perhaps placement of FRQ (and clock genes in general) into a functional context within a circadian system benefits from considering these properties. For instance, if the residual rhythmicity in *frq*-less strains derives from low amplitude, uncompensated or damped oscillations, then perhaps an entraining cycle will bestow an amplifying effect so that circadian characteristics outside of those contributed by the transcription-translation feedback loop can be identified (Roenneberg

& Merrow 1998). *frq*⁹ is capable of entrainment in temperature cycles, indicating that in the absence of *frq* gene function a rhythm generator is at some level still functional (Merrow *et al.* 1999). Several laboratories have reported that FRQ-deficient strains fail to entrain in light cycles (Chang & Nakashima 1997; Merrow *et al.* 1999; Lakin-Thomas & Brody 2000) although there is one report of entrainment (Loros & Feldman 1986). These results suggest a function for the FRQ protein in light signal transduction in addition to regulation of rhythmicity. As described in §3, *frq* expression is light-sensitive and the response of *frq* to light correlates with phase-resetting (Crosthwaite *et al.* 1995). Though FRQ-deficient strains are not blind—on the molecular level some light responsiveness remains (Arpaia *et al.* 1993, 1995b)—there is no regulation of rhythmicity by light in the absence of *frq* gene function (Chang & Nakashima 1997; Merrow *et al.* 1999; Lakin-Thomas & Brody 2000).

Taken together these observations are consistent with at least two interpretations (figure 3; see also Morgan *et al.* 2001). First, based on physiological data, Pittendrigh proposed (1960) that circadian systems consist of an A and a B oscillator, the A being entrainable by light and generally controlling entrained phase of the organism (figure 3a). The B oscillator is uncovered when alternative non-photoc inputs are used, or as in the experiments described above (Loros & Feldman 1986; Aronson *et al.*

1994a; Merrow *et al.* 1999; Lakin-Thomas & Brody 2000), under some conditions when the FRQ protein is not expressed. Alternatively (figure 3b), the activity ascribed to FRQ could function as part of a sensory transduction pathway, in this case communicating light information, in addition to conferring circadian properties to one or more downstream rhythm generators (Roenneberg & Merrow 1998; Merrow *et al.* 2001). Consistent with this hypothesis, FRQ-deficient strains fail to entrain to light cycles (Chang & Nakashima 1997; Merrow *et al.* 1999; Lakin-Thomas & Brody 2000) while in FRQ-sufficient strains the entire light input pathway in *Neurospora* is modulated by the clock (Merrow *et al.* 2001). The primary distinction between the two possibilities is whether or not the *frq*-based transcription–translation feedback loop is an independent oscillator capable of generating self-sustained rhythmicity. Although these distinctions are useful for modelling, hypothesizing and generally designing experiments, in the end the composition of a circadian system may not be definable as discrete oscillators but rather as modules that can be described by the physiology they contribute to the entire system which has physical responses indicative of an oscillator. In all of the molecular genetic model systems a transcription–translation feedback loop appears to be critical for conferring robust circadian periodicity and temperature compensation.

5. WHAT CAN THE STUDY OF CIRCADIAN OUTPUT PATHWAYS TELL US ABOUT THE FUNCTION OF THE CLOCK?

Organisms use their circadian clocks to regulate temporally a wide variety of cellular activities. The study of the flow of information from an oscillator to target output genes or proteins serves to: (i) identify components of the cell that are regulated by the clock in order to understand the role of rhythms in the life of the organism, and (ii) provide a route to study clock signalling mechanisms by tracing the regulatory circuit from the output back to the oscillator. Studies of circadian output pathways are also yielding information regarding the complexity of the time-keeping mechanism, including feedback of output onto input pathways and/or the oscillator(s), and the existence of multiple oscillators used to control different outputs. Thus, a complete understanding of the circadian system will require a detailed description of how circadian oscillators signal time information to regulate diverse output pathways.

To describe circadian output pathways in *Neurospora*, genes that are rhythmically expressed (i.e. controlled by the clock) but that do not affect oscillator function when inactivated were first targeted for isolation and the term ‘clock-controlled genes’ (*ccgs*) was coined to describe them (Loros *et al.* 1989; reviewed in Bell-Pedersen 2000). To date, eight *ccgs* have been identified as part of the output pathways by directed approaches (Loros *et al.* 1989; Bell-Pedersen *et al.* 1996c) and expression of several additional genes has been shown to be rhythmic with circadian periods (D. Bell-Pedersen, unpublished data; Lauter & Yanofsky 1993; Arpaia *et al.* 1995a). Aside from gene expression, a number of other clock outputs have been described in *Neurospora*, including oscillations in small

molecules (for a review, see Lakin-Thomas *et al.* 1990; Ramsdale & Lakin-Thomas 2001).

All the known *Neurospora ccgs* peak in transcript accumulation in the late night to early morning but they differ in overall expression levels and amplitude of the rhythm (Bell-Pedersen *et al.* 1996c). Because the *Neurospora* circadian clock provides an endogenous signal to regulate conidiation on a daily basis, and the peak in expression of the *ccgs* roughly correlates with the time of conidiation, it was anticipated that the *ccgs* would be associated with this developmental process. However, the levels of several *ccgs* are not induced during conidiation, suggesting that the clock governs more than just terminal differentiation (Bell-Pedersen *et al.* 1996c). Indeed it was found that *ccg-7* encodes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis and gluconeogenesis (Shinohara *et al.* 1998), *ccg-12* encodes copper metallothionein, involved in metal storage and detoxification (Bell-Pedersen *et al.* 1996c) and *ccg-9* encodes trehalose synthase, important for stress protection (Shinohara *et al.* 2001). Furthermore, *ccg-4* encodes a mating-type-specific pheromone (P. Bobrowicz, W.-C. Shen, L. Morgan, D. Bell-Pedersen and D. Ebbole, unpublished data), supporting a role for the *Neurospora* circadian clock in some aspects of the sexual developmental cycle. Thus, even in this relatively simple eukaryote, the output pathways appear diverse.

The most highly characterized *Neurospora ccg* is the *eas* (*ccg-2*) gene that encodes a hydrophobin important for spore dispersal (Bell-Pedersen *et al.* 1992; Lauter *et al.* 1992). The *eas* (*ccg-2*) gene is transcriptionally regulated by the circadian clock (Loros & Dunlap 1991) implicating *cis*-acting regulatory elements mediating temporal control. Dissection of the *eas* (*ccg-2*) promoter localized a positive activating clock element (ACE) to within a 45 bp fragment, found to be distinct from other elements regulating its expression (Bell-Pedersen *et al.* 1996a). Using an unregulated promoter–reporter system, the ACE was shown to be sufficient to confer high amplitude rhythmicity on the reporter gene. Protein factors present in *Neurospora* nuclear extracts have been shown to bind specifically to the ACE (Bell-Pedersen *et al.* 1996a) and experiments are underway to purify the factors in order to trace the signalling pathway back to the oscillator component(s) that controls *eas* (*ccg-2*) rhythmicity.

Circadian regulation of *eas* (*ccg-2*) appears to be through positive activation by the clock. Deletion of ACE results in constant low level transcript accumulation over the course of the day, and the maximal level of factor binding to the ACE occurs at the time of day when *eas* (*ccg-2*) mRNA is at its peak (Bell-Pedersen *et al.* 1996a, 2001). Interestingly, examination of the *ccgs* for mRNA accumulation in a *frq*⁺ versus a *frq* strain demonstrates that some *ccgs* accumulate significantly higher levels of message when FRQ is present in cells than when FRQ is absent. Other *ccgs* show the opposite pattern of mRNA accumulation, indicating positive and negative regulation by the clock (D. Bell-Pedersen, unpublished data). These data provide evidence for different regulatory pathways from the FRQ-based oscillator controlling output gene expression. However, it is not yet known if any of the components of the FRQ-based oscillator transduce time

information directly to regulate rhythmicity of any of the known output genes.

In the screens for rhythmically expressed genes in *Neurospora*, only a few times of day were compared and the screens were not saturating. Furthermore, only *cgs* that accumulated message with a long period in the 29 h period *frq*⁷ mutant strain were further studied. In retrospect, this may have missed *cgs* that are regulated by oscillators that are independent of FRQ. Thus, the *cgs* probably represent only a small sampling of clock-regulated genes in *Neurospora*. Experiments are currently in progress using transcriptional profiling to determine the full extent of clock-regulation of gene expression (see § 8).

6. OTHER RHYTHMS IN FUNGI

When discussing the complexity of the *Neurospora* clock it may be useful to have a look at what is known about circadian rhythms and other biological timing mechanisms in fungi in general. Periodic growth patterns of fungi are easily observed in nature or on our table on over-ripe fruit. However, only a few of these fungi have been studied scientifically and none of them in much detail (for reviews, see Bell-Pedersen *et al.* 1996b; Ramsdale 1999). In many cases, the periodic pattern was found not to be due to an endogenous clock but rather required an alternation of light and darkness or temperature cycles (Lysek 1984; Ramsdale 1999). It is still not known if these fungi lack circadian rhythms entirely or whether it is just that growth and sporulation are not under circadian clock control in these species. The lack of an obvious clock phenotype does not necessarily mean that they do not possess biological oscillators that confer temporal control over the organism's activities. An example of an unusual endogenous rhythm is that of trap formation in the nematophagous fungus *Arthrobotrys oligospora* (Lysek & Nordbring-Hertz 1981). Here, traps to capture living nematodes are formed periodically with a period of about 42 h. This period is temperature compensated and is independent of the medium on which the fungus grows, indicating the presence of a clock that functions with a homeostatically regulated period. On the other hand, this rhythm is not entrained by a 24 h light–dark cycle—a hallmark of a circadian rhythm.

Is this example the odd one out or are there more surprises in store as far as the temporal regulation of fungal life is concerned? We do not know the answer at the moment, which also means that we do not know to what extent *Neurospora* is typical for filamentous fungi. Thus, it might be of interest to look for circadian rhythms in other filamentous fungi in more detail, in particular in well studied examples such as the *Aspergillus* species and in those fungi where genome sequencing projects are well underway. Indeed, a circadian rhythm in spore production has been identified in *Aspergillus flavus*. This rhythm is entrained by light–dark cycles and is temperature compensated (A. Greene, N. Keller and D. Bell-Pedersen, unpublished data). However, attempts to identify a similar rhythmic phenotype in the more amenable experimental system of *A. nidulans* have thus far been unsuccessful. An alternative approach to identify an endogenous clock in species that lack an easily detectable

phenotype involves searching for rhythmically expressed genes when cultures are grown under constant conditions. Experiments are currently underway in attempts to identify rhythmically expressed genes in *A. nidulans* using differential display and hybridization to known fungal clock-associated genes. Such analysis should allow comparisons between clocks within the fungal kingdom, and provide additional information regarding links between rhythmic phenomena and the specific biology of the organism.

7. MATHEMATICAL MODELLING

In attempts to describe the essential and important processes involved in the timing mechanism, minimal mathematical models have been created and used to make predictions about oscillator function. Biological models can range from pure qualitative descriptions of processes (via mathematical equations describing certain variables) to models showing detailed reaction kinetics of the underlying biochemical reaction pathways. In the following, we present a brief summary of different types of mathematical or reaction kinetic models that have been applied to the study of circadian rhythms in *Neurospora*.

A model that provides a mathematical description of phase resetting data from *Neurospora* with no correspondence to the underlying physiological processes was proposed by Lakin-Thomas *et al.* (1991). In this model, period and amplitude are genetically determined by the same parameter, and changes in amplitude compensate for changes in temperature. Three different sets of equations based on different oscillator mechanisms were shown to give similar predictions. The model predicts that a mutant with a long period has a weaker response to resetting stimuli than a short-period mutant, and this prediction was shown to be correct for several mutants with altered periods exposed to pulses of light or cycloheximide.

The amplitude model does not provide any details as to what the biological oscillator might be. A step closer to a molecular approach is the Roenneberg & Merrow (1998) model. These authors proposed that mutations in clock genes such as *frq* or the *Drosophila per* gene could lie in an input pathway to a central rhythm generator rather than in the rhythm generator mechanism itself. By model calculations the authors demonstrated that this is indeed possible and placed the central rhythm generator into an unknown 'black box' that still needs to be described. Furthermore, the model equates metabolic and temperature compensation and proposes a stabilizing effect of the transcription–translation feedback loop on metabolic noise in the system (Roenneberg & Merrow 1999). This model predicts that rhythmicity in the absence of *frq* gene function should be insensitive to light and this has been demonstrated in *frq*-null strains (Lakin-Thomas & Brody 2000; Merrow *et al.* 1999).

The first model that proposed a mechanism for a biological oscillator was published over 30 years ago by Goodwin (1965). The model (figure 4) proposed that the oscillator is a negative feedback loop in which the period-determining step is degradation of the intermediates. A longer degradation time of the clock protein gives a prolonged inhibition of transcription of the clock gene, which then results in longer period length. The importance

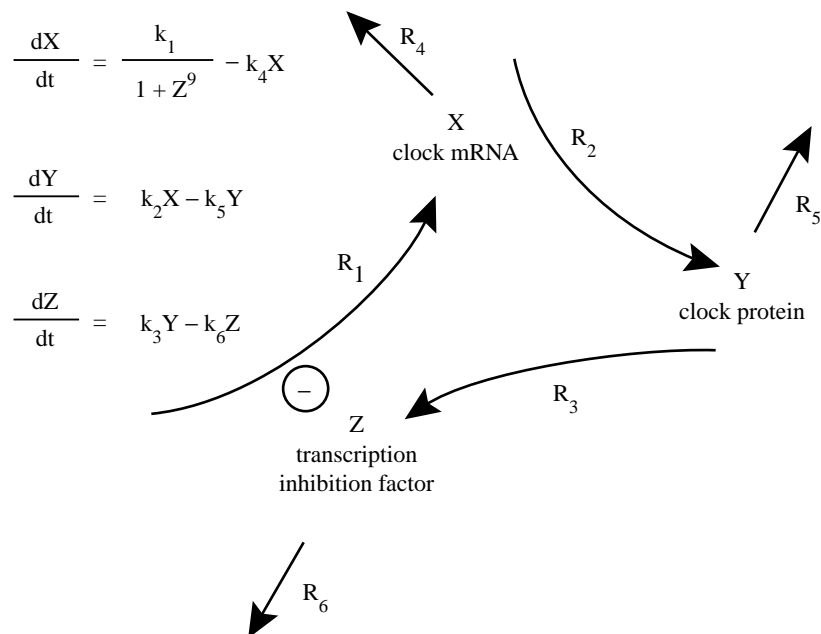


Figure 4. The Goodwin (1965) model. A clock mRNA is transcribed from a clock gene. A clock protein is translated from the clock mRNA, which is modified before it goes into the nucleus and inhibits the transcription of the clock gene. X, Y and Z represent the concentrations of the three state variables: clock mRNA, clock protein and transcription inhibition factor. R_1 , R_2 and R_3 are reactions representing the synthesis of the three state variables, while R_4 , R_5 and R_6 represent the degradation reactions. k_1 to k_6 are rate constants associated with their respective reactions (R). dX/dt is the rate of change of X. The negative sign indicates the inhibition of the transcription reaction by Z.

of degradation, and thus the stability of the intermediates, has now been indicated for *Neurospora* (Liu *et al.* 2000). Calculations using the Goodwin model have been successfully compared with experimental results, including resetting by temperature, cycloheximide and light; and *frq* mRNA and FRQ protein levels have also been modelled (Ruoff *et al.* 1999a,b; Vinsjevsk *et al.* 2001).

The most detailed model describing the *Neurospora* circadian oscillator was proposed by Goldbeter (Leloup *et al.* 1999; Gonze *et al.* 2000). The model describing the *Neurospora* clock proposes a negative feedback mechanism for the oscillator and is based on the Goodwin model, but the details differ. The model successfully simulates light regulation and entrainment of *frq* RNA and FRQ protein rhythms. The model also predicts that oscillations of *frq* RNA should continue in constant light and could be forced into chaos in light–dark cycles, but these phenomena have not been observed.

To sum up, do the models have something in common? All models have at least one feedback loop, which has been proposed for circadian oscillators in organisms ranging from cyanobacteria to *Neurospora*, *Drosophila* and mammals (Aronson *et al.* 1994b; Ishiura *et al.* 1998; Dunlap 1999; Green & Tobin 1999). The mathematical formulae in the models are very different, and yet they all produce good descriptions of the behaviour of the *Neurospora* circadian system. This may indicate that all of the models are oversimplified in that they do not contain all the components of a complex system. Although a system may be complex, it is not necessarily the complex models that give the best predictions. Simple models give simple predictions that can easily be tested. Detailed models give much better quantitative descriptions but the details can hide the most important processes.

Mathematical models are not reality, just tools for understanding the biology, and a complex system needs several tools to solve its problems. The variety of models for *Neurospora* is an advantage: their predictions might confirm each other, or they might differ and suggest new experiments to distinguish between them.

What are the requirements for successful models? Such models should describe the basics of the clock mechanism and account for the experimental results. They should also be able to predict properties of the mechanism that are not possible to predict by sheer intuition, and thus provide guidelines and rationales for further experimental studies. The next step forward will rely on insertion of real parameter values for the different processes (Lakin-Thomas & Johnson 1999). Such information will give the correct rate constants for the different processes and, in the end, the dialogue between theory and experiment will provide a detailed mechanism of the circadian clock.

8. WHAT CAN NEW TECHNOLOGIES TELL US ABOUT THE COMPLEXITY OF THE *NEUROSPORA* CLOCK?

Although genetic approaches have been successful in identifying components of the circadian system, our use of genetics in the study of clocks to date has been largely restricted to brute force screens and clock mutants without major pleiotropic effects on other cellular processes (Dunlap 1993). This may be due to the bias of investigators in choosing ‘healthy’ mutants for further study (Nakashima & Onai 1996; Lakin-Thomas 2000) or these mutants may be exceptional in that they result from alterations in genes that lack redundancy of function (Dunlap 1996; Roenneberg & Merrow 1998). Only recently have clock-affecting loci in other organisms been

identified by screening mutant strains that express a reporter gene at the wrong time of day (Millar *et al.* 1995; Golden *et al.* 1997). No targeted selections, and only limited conditional mutant screens and suppressor screens, have been described in *Neurospora* (Chang & Nakashima 1998). One potentially powerful screen is to obtain non-rhythmic mutants in FRQ-null strains under conditions that permit rhythmicity. This will allow the identification of components of putative additional oscillators. Undoubtedly, development of such screens will be necessary for the continued evolution of the field.

A global understanding of the extent to which the clock influences and is influenced by cellular processes will come with transcriptome, proteome and metabolome studies where the expression of genes and their products can be correlated with circadian phenotypes. Such genomic approaches may also provide useful information regarding the extent to which genes that appear redundant at the genetic or functional level cover similar functions or are involved in parallel pathways. In this regard, experiments using transcriptional profiling with DNA microarrays are underway in several laboratories, and expressed sequence tags sequencing of time-of-day specific *Neurospora* cDNA libraries (Bell-Pedersen *et al.* 1996c) is ongoing (<http://www.genome.ou.edu/fungal.html>). Together these studies will provide an unbiased means to identify clock-associated genes that are regulated rhythmically at the transcriptional level and allow a determination of the prevalence of rhythmic gene expression in the cell. Furthermore, these studies will shed light on the complexity of the clock and allow characterization of output pathways arising from one or more oscillators. These analyses will probably uncover links between the regulation of development, metabolism and rhythmicity in *Neurospora*. While clock genes can be used as tools to isolate remaining clock components and investigate the nature of input and output pathways, the quantity of data accumulating will increasingly demand the use of dynamic modelling programs to validate hypotheses about the clock mechanism.

9. HOW COMPLEX IS THE *NEUROSPORA* CLOCK?

Before attempting to answer this question, we need to define what we mean by 'complex'. It was notable that during the entire meeting of which these *Transactions* are a record, no one was foolhardy enough to attempt a definition. As a working heuristic, simple things have few component parts and complex things have many. We will consider the 'manyness' of the *Neurospora* clock at several levels of organization. (i) How many gene products are involved in the clock mechanism? (ii) How many feedback loops interact to construct the complete clock mechanism? (iii) How many spatially independent clock mechanisms might there be in the whole organism?

(a) Multiple clock genes

How many gene products contribute to circadian timing in *Neurospora*? Mathematical modelling (see § 7) demonstrates that only a few components are necessary to build a temperature-compensated oscillator. Unfortunately, nature is probably not as parsimonious as the

mathematicians. *Neurospora* does not seem to have multiple copies of the canonical clock genes (defined as those genes whose products are proposed to be major components of a rhythm-generating transcription–translation feedback loop), unlike mammals which have several *per* genes. There is no evidence that there is more than one *frq* gene in *Neurospora crassa* (M. Merrow, unpublished data) and the *wc* genes, *wc-1* and *wc-2* are not redundant (Crosthwaite *et al.* 1997). There are, however, more than 20 other clock-affecting genes (for a review, see Lakin-Thomas 2000; Morgan *et al.* 2001) that have been shown to affect the period of the rhythm. Two of these genes, *cel* and *chol-1*, are defective in lipid metabolism and have major effects on the period and temperature-compensation of the conidiation rhythm. This and other evidence may indicate that metabolic pathways are intimately involved in the clock mechanism (Roenneberg & Merrow 1999) and, if so, then there may be a large number of other gene products with the potential to affect rhythmicity. It is likely that only a few gene products will have major effects on the kinetics of the rhythm but there may be many that have minor effects.

(b) Complex feedback loops

When we move beyond individual gene products, we can ask how these gene products are organized into a clock mechanism. There may be more than one oscillator loop constituting the complete clock mechanism, and there may be feedback from the oscillator(s) onto input pathways and/or feedback from output pathways onto the oscillator mechanism. It appears that even a relatively simple organism, such as *Neurospora*, shows evidence of this kind of complexity.

One indication of certain kinds of complexity in oscillatory systems is the presence of phase transients after a resetting stimulus. Transients can be produced by hierarchical systems in which a master oscillator entrains a slave oscillator, as in Pittendrigh's A and B oscillator system (Pittendrigh 1960), or they can be produced when a system consists of two or more mutually coupled oscillators, or a limit cycle that slowly returns to its preferred trajectory (Winfree 1980). Transients after a phase shift have never been reported in *Neurospora*. This may indicate either that the system is relatively simple, that multiple oscillators are tightly coupled, or that the right experimental conditions have not yet been tested.

Are there multiple oscillators contributing to the circadian system of *Neurospora*? The persistence of rhythms in the absence of *frq* (see § 4), demonstrates the existence of at least one other functional oscillator, which has been called the FLO, or *frq*-less oscillator (Iwasaki & Dunlap 2000). The relationship between the *frq* loop and the FLO has not been established (see also Morgan *et al.* 2001). There is no evidence for any light-insensitive rhythms in wild-type strains, which might indicate an oscillator completely independent of *frq*. If we assume that the FLO is functional in both *frq*⁺ and *frq*-null strains, then the data suggest that the *frq* loop and the FLO are normally coupled, and *frq* and *wc* functions are required for light-sensitivity of the FLO (see § 4). The activity of components of the light input pathway, particularly the WC proteins, is rhythmic (Merrow *et al.* 2001). This may indicate either that these components are part of a central

oscillator feedback loop or that there is feedback from a central oscillator onto the light input pathway.

Do output pathways feed back on the central oscillator? Mutations in known *Neurospora* clock-controlled genes have not been shown to affect the period of the rhythm (Bell-Pedersen *et al.* 1992; Lindgren 1994; Shinohara *et al.* 2001). However, even mutations that abolish conidiation at early stages do not abolish aerial hyphae formation (Martens & Sargent 1974; A. Correa and D. Bell-Pedersen, unpublished data) and no mutants are known that are specific to aerial hyphae formation. There are other output pathways besides conidiation in *Neurospora*, as shown particularly by the existence of clock-controlled genes that are not conidiation-related (see § 5). Recently, Ramsdale and Lakin-Thomas (Ramsdale & Lakin-Thomas 2001; Lakin-Thomas *et al.* 2001) reported circadian rhythms in the neutral lipid diacylglycerol (DAG) in *Neurospora*. DAG levels are high in a *chol-1* mutant strain that has a long period of 60 h under some conditions, suggesting that a correlation might exist between DAG levels and period. The addition of membrane-permeable DAG and inhibitors of DAG kinase further lengthened the period in this strain, hinting that DAG may feed back on the time-keeping mechanism to affect the period. These data provide the first suggestion of feedback from an output to an oscillator in *Neurospora*.

(c) *Spatial complexity*

The discussion above dealt with events at the molecular level and within what we might call a 'unit of space', which would be a single cell in a multicellular organism. A different kind of complexity can be considered when we look at a whole organism: how many spatially independent clocks are there in *Neurospora*, and is there a hierarchical organization? In a classic paper, Dharmananda & Feldman (1979) demonstrated that there are independent light-sensitive circadian oscillators in all parts of the fungal mycelium and phase information is not readily transferred from front to back of the fungal colony. Winfree & Twaddle (1981) demonstrated that phase information is also not transferred laterally in the mycelium. *Neurospora* is coenocytic, meaning it has no solid cell walls between discrete cells but rather allows a flow of cytoplasm and organelles through septal pores along the hyphae. The barrier to a flow of phase information has not been identified. However, older mycelia develop very large vacuoles that probably constrict the nuclear flow that occurs readily at the growing tips.

Might there be different oscillator mechanisms in different regions or within the same unit of space in *Neurospora*? Lakin-Thomas and colleagues (Lakin-Thomas *et al.* 2001; P. L. Lakin-Thomas, J. C. Thoen and V. D. Gooch, unpublished data) have reported rhythms in both the determination and differentiation stages of conidiation, and these two rhythms are not always tightly coupled. It is possible that these two rhythms might be driven by spatially separate oscillators (Lakin-Thomas *et al.* 2001). The rhythm in DAG levels (Ramsdale & Lakin-Thomas 2001) is also not tightly coupled to the rhythm in conidiation bands, and this may also be an indication of the existence of multiple oscillators in the mycelium.

Does the *Neurospora* circadian clock interact with non-circadian timers, such as ultradian clocks and the cell cycle? On one level, the circadian system of *Neurospora* seems to be quite fundamentally different from that of other eukaryotes studied so far: the circadian clock operates in a very fast growing mycelium. The circadian–infradian rule, one of the long-held paradigms in chronobiology (Edmunds 1988)—which states that circadian clocks are found only in organisms with infradian growth (i.e. with a doubling time longer than the circadian period)—has always disregarded that *Neurospora* is an example to the contrary. However this rule seems to be strictly adhered to in other eukaryotes. For example, in fission yeast cultures growing at a low rate, the circadian rhythms in cell division and gene expression are immediately lost as soon as the generation time decreases below the circadian period (Kippert 1997). During faster growth, an ultradian clock exerts temporal control over many aspects of cell physiology (Kippert & Hunt 2000). In fission yeast, as in many other organisms, circadian and ultradian clocks seem to be strictly alternative timing modes.

The question arises whether there may be an ultradian clock in the growth front of a fast-growing mycelium, operating simultaneously with the circadian clock of *Neurospora*. There is one indication of this from the literature. Fletcher & Trinci (1981) observed ultradian oscillations in oxygen uptake in *Neurospora* cultures synchronized with 2'-deoxyadenosine. These are very reminiscent of oscillations in energy metabolism, induced by the same means, which have been observed in several micro-organisms and in many cases have been shown subsequently to be the output of a temperature-compensated ultradian clock (Kippert 1997). In contrast to its cousin *Aspergillus*, well studied in regulation of the duplication cycle (Harris 1997), knowledge about cell cycle regulation in the growing tips of *Neurospora* is rather rudimentary. The possible coexistence of circadian and ultradian clocks in the same cytoplasm would add an interesting twist to the complexity of biological timing in *Neurospora*.

(d) *Conclusion*

In summary, then, how complex is the *Neurospora* clock? Complexity can be measured in the number of gene products playing major roles in sustaining and regulating rhythmicity, and the number of semi-independent loops and oscillators. The *Neurospora* circadian system is probably more complex than it needs to be, if we were to take the ideal minimal mathematical oscillator model as a baseline. On the other hand, it is certainly not as complex as the circadian system of a vertebrate, in which there may be multiple pacemakers in the brain, eye and pineal, interacting with each other and driving slave oscillators in peripheral tissues. The complexity of the *Neurospora* clock may reflect the influences of regulatory loops controlling metabolic pathways and the adaptations of environmental sensors to external and internal noise.

Dedicated to Professor David Saunders on the occasion of his retirement, in recognition of his years of insightful research and masterful teaching of the classics and basics of circadian rhythmicity. Many thanks to Fred Kippert for his substantial and

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